# Chapter 5

## A lesion mimic phenotype in tomato obtained by isolating and silencing a *Lls1* homologue

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**Summary**

**Lesion mimic phenotypes serve as a tool to study the regulation of cell death in plants. In order to obtain a tomato lesion mimic phenotype, we used the conservation of the lethal leaf spot 1 (*Lls1*) genes between plant species. The tomato *Lls1* homologue was cloned, sequenced and analyzed. It showed high conservation at the protein and at nucleotide level compared to the genes from maize and *Arabidopsis*. Using virus induced gene silencing, we obtained a phenotype resembling the *lls1* mutant in maize. Lesion formation, lesion spreading and light dependence of the tomato *lls1* phenotype are strong indications for the functional conservation of the *Lls1* gene between the dicotyledonous tomato and the monocotyledonous maize.**

## Introduction

Programmed cell death (PCD) in plants and animals is a process by which cells are selectively eliminated in a controlled way (Buckner *et al.*, 1998; Jabs, 1999). In plants, PCD occurs during development and as a localized response associated with pathogen attack, known as the hypersensitive response (HR).

There are three functionally distinct phases of PCD: induction, effector and degradation phase (Jabs *et al.*, 1996; Jabs, 1999; Kroemer *et al.*, 1995). During the induction phase the cells receive death signals, which could include absence or reduction of survival factors, perturbance of the normal metabolic pathways or cytotoxic agents as reactive oxygen intermediates (ROI) (Jabs, 1999). ROI are known to mediate the induction of cell death in both animals and plants (Chen *et al.*, 1993; Cimino *et al.*, 1997; Jabs *et al.*, 1997; Lamb and Dixon R.A., 1997; Lander, 1997; Schulze-Osthoff *et al.*, 1997).

In plants, ROI are associated with HR cell death (McDowell and Dangl, 2000) and with photooxidation (Reinbothe *et al.*, 1996). When a resistant plant is challenged by a pathogen and the plant recognizes the invader, this increases the level of ROI (Grant and Loake, 2000; McDowell and Dangl, 2000). All photosynthesizing organisms need to protect their cells from the danger of photooxidation. Plants evolved protective mechanisms to overcome the problem of excessive production of ROI (Reinbothe *et al.*, 1996) and to degrade the already produced dangerous reactive oxygen molecules by antioxidant enzymes (Jabs, 1999).

The ROI have been shown to play a key role in certain lesion mimic mutants (Jabs *et al.*, 1996; Molina *et al.*, 1999). Lesion mimic mutants are known mainly from *Arabidopsis* and maize and they exhibit disease-like symptoms (HR-like) in the absence of pathogens (Buckner *et al.*, 1998). They are powerful tools for studying the HR-like cell death in plants. A number of the genes responsible for the lesion mimic phenotypes, such as *lsd1, les22* and *lls1*, have been cloned and the functions of the encoded proteins established or predicted. The *Lsd1* gene encodes a zinc finger transcription factor, which is proposed to be part of a signaling pathway for the induction of the CuZn superoxide dismutase, an antioxidant enzyme (Dietrich

*et al.*, 1997; Kliebenstein *et al.*, 1999). The maize lesion mimic phenotype *les22* is due to a null mutation in the uroporphyrinogen decarboxylase gene, which encodes an enzyme in the porphyrin pathway in plants (Buckner *et al.*, 1998; Hu *et al.*, 1998). The loss of function of the uroporphyrinogen decarboxylase gene leads to an accumulation of uroporphyrin. When photo-excited, uroporphyrin forms singlet oxygen and this can initiate cell death.

The lethal leaf spot 1 (*lls1*) mutation in maize behaves as a recessive lesion mimic (Ullstrup and Troyer, 1967). The lesions start as necrotic spots and enlarge with a typical zonal pattern. The tissue between spots wilts and dries shortly after the lesions reach the maximum size of 1-2cm. Subsequently, the whole leaf dies. The *Lls1* gene from maize has been cloned and sequenced. Analysis of the predicted amino acid sequence revealed two conserved motifs the Rieske type center and the mononuclear nonheme Fe-binding site (Gray *et al.*, 1997). These motifs, are known from the α-subunit of all aromatic ring-hydroxylating (ARH) dioxygenases. Between ARH dioxygenases, these two motifs show high conservation at the protein and DNA level. The spacing between them is also conserved. It was suggested that the possible function of the LLS1 gene product is to degrade a phenolic mediator of cell death that is triggered under oxidative conditions (Gray *et al.*, 1997). Using a database search, (Gray *et al.*, 1997) found *Arabidopsis* expressed sequence tags (ESTs) showing 70% homology with the *Lls1* cDNA from maize.

Tomato is a genetically well-characterized plant species (Tanksley *et al.*, 1992). In tomato, there are a number of resistance genes cloned and characterized, which confer resistance associated with an HR (Hammond-Kosack and Jones, 1996). The mechanism leading to HR cell death is still not well characterized. Lesion mimic mutants serve as tools to study this process in plants. The aim of this study was to clone the tomato *Lls1* homologue and to generate a lesion mimic mutant in tomato. We used the proposed conservation of the *Lls1* gene between monocotyledons and dicotyledons. For cloning of the tomato *Lls1* homologue heterologous hybridization was applied with *Arabidopsis* and maize *Lls1* probes. Subsequently, the tomato *lls1* like phenotype was obtained by virus-induced gene silencing (VIGS).

## Experimental procedures

### *Primers for amplifying the* LLS1 *gene*

For amplifying the Arabidopsis *Lls1* fragment the following primers were used: forward 5'TTGCAATGTTCGTATCATGGAT3' and reverse 5'TGGGAAGGATC AGATACATTTTCC3'. For amplifying the maize fragment forward primer 5'TTGCAGTGCTCGTATCACGG3' and reverse primer 5'TATATGGGACGGAT CAGAGACG3' were used.

### *Southern blot analysis and l library screening*

All plant DNA isolations were performed using the CTAB DNA isolation protocol (van der Biezen *et al.*, 1996). Southern blot analyses were performed with 8μg of

genomic DNA (from each of the studied plant species) digested with *Eco*RV. A tomato genomic library in the λ phage EMBL3 SP6/T7 was screened with *Arabidopsis* and maize probes to obtain the *Lls1* homologue. The hybridizations were performed at 57°C; followed by two washing steps with 2x SSC for 5 min at 57°C and with 0.1 x SSC and 0.5% SDS for 3 min at room temperature. In order to subclone the positive λ clones, the restriction enzymes *Bgl*II and *Hin*dIII were used. Cloning of the fragments was according to standard techniques (Sambrook *et al.*, 1989).

*RNA analysis*

For the total RNA isolation the 'RNeasy Plant Mini Kit' (Qiagen) was used. Northern blot analyses were performed with 15µg total RNA for each of the studied plants. Hybridizations were done at 65°C with subsequent washing with 2x SSC for 5 min at 65°C and two more washing steps with 0.1 x SSC and 0.5% SDS at 65°C and 50°C for 10 min each.

The 5'RACE and RT-PCR were performed on total RNA from the tomato cultivar Moneymaker. For the 5'RACE, the '5'/3' RACE Kit' (Roche Molecular Biochemicals) was used with primers for the first strand synthesis 5'ATGACCATCCATGATATGAACA3' and for the first and second PCR reactions 5'ACTGGGTACCAATGATCTCTCC3' and 5'TAGCTGTTGTTGTTGTTGGA GG3' primers were used, respectively.

For the RT-PCR amplification the 'Omniscript RT Kit' (Qiagen) was used with a first strand synthesis primer 5'TCTTGGAGAGATCATTGGTACC3' and with a second strand reaction primer 5'ATGACCATCCATGATATGAACA3'.

*Sequencing and sequence analysis*

The ALFexpress II system from Amersham Pharmacia Biotech was used for sequencing. For analysing the tomato *Lls1* cDNA contig, the 'Sequencher 3.0' software was used. For multiple alignment of the mRNA, protein sequences and for building the phylogenetic trees the 'Clustal W' program (Saitou and Nei, 1987; Thompson *et al.*, 1994) was used. Analyses of the domain structures of the LLS1 proteins were performed on the ProDom (Altschul *et al.*, 1997) protein domain database server in Toulouse, France. Blast searches were done at NCBI and TAIR.

*Silencing of the tomato LLS1 homologue*

The 250bp tomato *LLS1* RT-PCR product (see above) was cloned into pGEMTeasy (Amersham Parmacia Biotech) and subsequently was subcloned as a *Not*I restriction fragment in to pGR106 (Angell and Baulcombe, 1999). The resulting plasmid pGRtlls6/7 and pGR106 were introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation together with pSoup (Hellens *et al.*, 2000).

The transformation of tomato cultivar Moneymaker was carried out by *A. tumefaciens* at the six leaves stage by three punches on the stem with a sterile toothpick covered with *Agrobacterium*. The inoculated plants were kept at high

humidity for 3-4 days. *Nicotiana benthamiana* plants were inoculated in a similar way as tomato plants at six leaves stage, but as punches on the 2nd leaf instead of the stem.

## Results

*Cloning of the tomato LLS1 homologue*

In order to clone the *Lls1* homologue from tomato, we used high sequence identity, above 70%, between the maize and the *Arabidopsis Lls1* cDNAs. The two conserved motifs of the LLS1 proteins, the Rieske type centre and the mononuclear nonheme Fe-binding site, show higher homology at the protein level, above 90% (81% identity). We used these motifs to design PCR primers based on *Arabidopsis* and maize genomic templates. These amplified fragments were used as probes in Southern blot analysis. The Southern blot revealed one or two *Lls1* homologues sequences in all tested species (Figure 1). Only one tomato genomic fragment hybridised with the *Lls1 Arabidopsis* probe, suggesting one copy of the *Lls1* homologue in tomato.

Screening the tomato genomic library with the *Lls1 Arabidopsis* probe resulted in six positive λ clones. Only one of the clones cross-hybridised with the maize *Lls1* probe and this clone was partially sequenced. The tomato genomic sequence showed significant homology to maize and *Arabidopsis* genomic *Lls1* sequences, above 60% and 65%, respectively, suggesting we had cloned an authentic tomato *Lls1* homologue. Tomato expressed sequence tags (ESTs) were found, which were 100% identical to parts of the tomato *Lls1* genomic sequence. The ESTs were derived from different tomato cDNA libraries, including libraries from 7-days-old germinating seedlings, callus, ripe fruits and leaves which suggests expression throughout plant development.

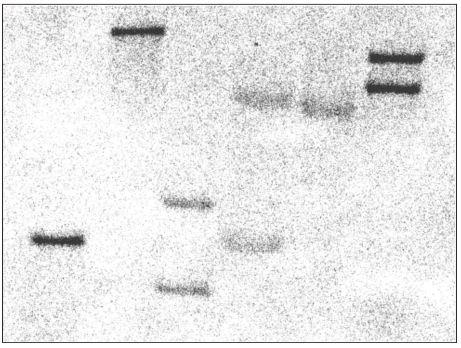


**Figure 1.** *Southern blot analysis of genomic DNA isolated from Brassicaceae and Solanaceae species*.

All DNAs were restricted with *Eco*RV. The *Lls1* fragment from *Arabidopsis* was used as a probe. The fragment includes the DNA regions coding for the Rieske type centre and the mononuclear nonheme Fe-binding site.

1.*Arabidopsis thaliana* 2.*Sinapis alba* 3.*Brassica rapa* 4.*Thilaspi arense* 5.*Lycopersicon esculentum* 6.*Lepidium sativum*.

In order to construct a full-length tomato *Lls1* mRNA, the cDNA clone (No. cLER7H9) was sequenced. The clone corresponds to EST254701. The 5' end of the tomato *Lls1* mRNA was confirmed by 5'RACE. The resulting cDNA sequence containing the complete coding region revealed a tomato *Lls1* transcript of 1717 nucleotides (No. AF321984). Translation of the tomato *Lls1* mRNA results in a 537aa protein. The predicted molecular mass of the protein is 60.7kDa, similar to the predicted molecular weight of the maize LLS1 protein 58kDa (Gray *et al.*, 1997).

*Sequence analyses of Lls1 homologues*

In order to study further the relation of the LLS1 proteins with the proteins that show considerable homology in a blast search, we analysed their domain structure and built phylogenetic trees.

The LLS1 proteins from tomato and maize have a Rieske iron-sulphur domain of about 80aa and a second domain of 170aa that includes a mononuclear nonheme Fe-binding site at its N-terminus (Figure 2a). The distance of about 60aa between both domains is preserved. The LLS1 proteins share the same domain structure with the TIC55 precursor protein from *Pisum sativum* and a predicted Rieske type protein from *Synechocystis sp.* (slr1747).

We built phylogenetic trees using the Neighbour Joining method (Saitou and Nei, 1987). All of the proteins used to build the phylogenetic trees contain the Rieske type centre and the mononucleotide nonheme-Fe-binding site. The alignment of the entire proteins (Figure 2b) or the alignment only of the Rieske domains (data not shown) resulted in a similar tree topology. The LLS1 proteins formed one group. The Rieske type protein from *Synechocystis sp.* is also included in this group, suggesting a possible functional homologue. The TIC55 protein that shares the same domain structure with LLS1 proteins shows similar output distances to the group of the LLS1 proteins as CAOes. The two bacterial proteins, phenoxybensoate dioxygenase α-subunit from *Pseudomonas pseudoalcaligenes* (Dehmel *et al.*, 1995) and lignin biphenyl-specific *O*-demethylase from *Sphingomonas paucimobilis* (Sonoki *et al.*, 2000), did not show significant divergence from the LLS1 group than the other proteins shown in Figure 2b.

*Tomato LLS1 gene silencing and phenotype*

VIGS was used to study the phenotype of the tomato *Lls1* homologue (Figure 3). An RT-PCR product representing 250bp of the tomato *Lls1* gene was cloned in the PVX vector and introduced into tomato plants and *N. benthamiana* via in planta *A. tumefaciens* transformation. In tomato, the first PVX symptoms were observed 10-13 days post inoculation and 7-9 days later, the first *lls1* like symptoms appeared (Figure 3A1 and 3A2). In *N. benthamiana,* virus symptoms appeared 10 days after inoculation and the first *lls1*-like symptoms were visible 6-7 days later (Figure 3B). All lines inoculated with pGRtlls6/7 (pGR106::tomato *Lls1* RT-PCR product) showed the same lesion pattern. In both tomato and *N. benthamiana,* control

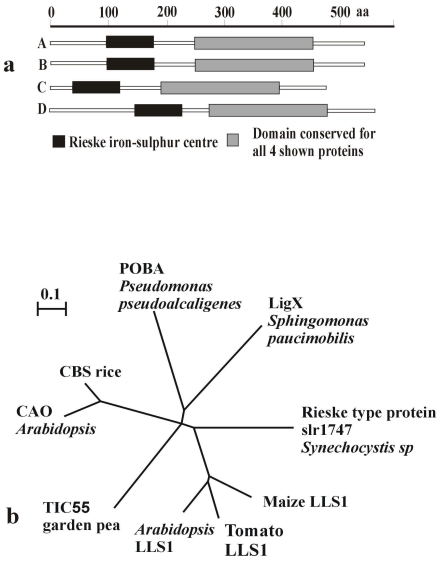


**Figure 2**. *Sequence analysis of the tomato Lls1 gene*.
(a) Protein domain structure of (A) tomato LLS1; (B) maize LLS1; (C) *Synechocystis sp*., hypothetical protein (slr1747) (D) TIC55 garden pea.
(b) Phylogenetic relationships between LLS1 proteins, TIC55 protein from garden pea, hypothetical protein (slr1747) from *Synechocystis,* CAO *Arabidopsis,* CBS rice, phenoxybensoate dioxygenase α-subunit (POBA) *Pseudomonas pseudoalcaligenes* and lignin biphenyl-specific O-demethylase (LigX) *Sphingomonas paucimobilis*. The bar shows the output distance, which is given as substitutions per site.

inoculations with empty PVX vector resulted only in symptoms typical for PVX infections (Figure 3A3 and 3B2).

Northern blot analysis did not detect the *Lls1* transcript in the leaves of tomato plants inoculated with the silencing construct (Figure 4). The *Lls1* transcript could be detected in RNA isolated from the leaves of control plants, which are either not inoculated or inoculated with the PVX construct without insert. The 225bp 5'RACE product was used as an *Lls1* probe for the Northern blot analysis. The sequence of the 5'RACE product does not overlap (80bp upstream) with the sequence of the RT-PCR product used for silencing.

VIGS with *Lls1* tomato DNA sequence in tomato and in *N. benthamiana* resulted in lesion symptoms similar to the reported maize mutant. The lesions increased in size with the zoned pattern typical for the *lls1* maize mutants (see the enlarged panels on Figure 3A1 and 3B3). In tomato, the lesions appeared first at the tip of the leaves, developmentally the oldest part, the tissue between the lesions dried rapidly and finally the whole leaflet wilted and died (Figure 3A2). The leaflets from one and the same compound tomato leaf died simultaneously. After the tissue of the leaf had become necrotic and dry, the leaf detached from the stem. After the first symptoms were observed (2-3 weeks), the whole plant leaf tissue died. The stem and the fruits were not affected (Figure 3A1).

As in maize, the tomato *lls1* phenotype is light dependent. In our experiments, 48 hours of darkness delays the formation of *lls1* silencing symptoms (Figure 3C). After the plants were returned to the light conditions in the greenhouse, they continued to develop the symptoms, though they kept the 48 hours delay compared

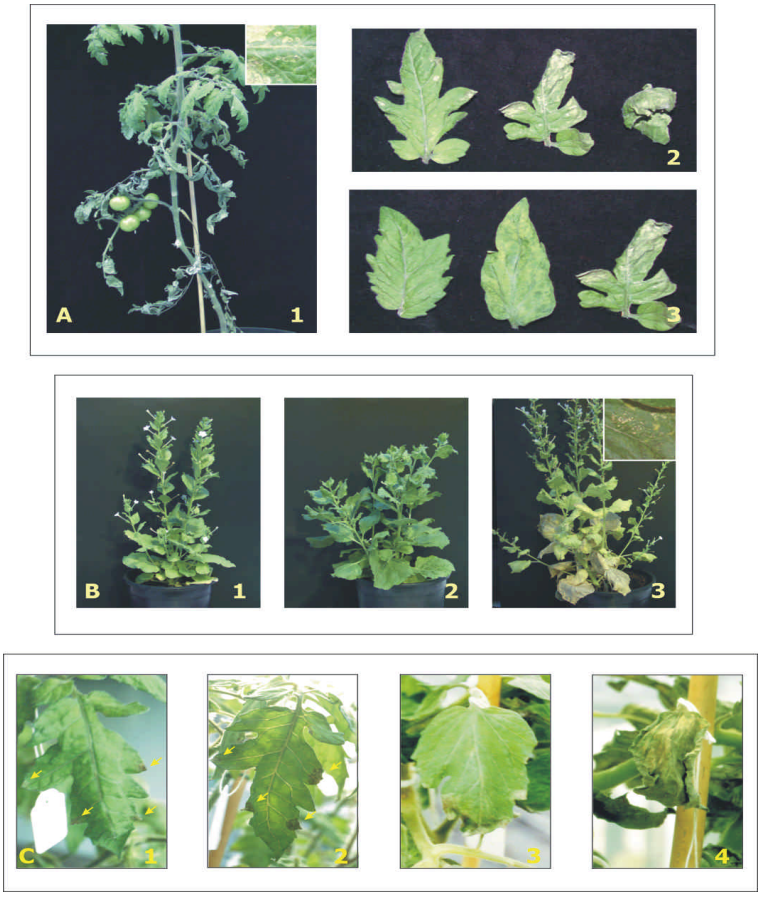


**Figure 3.** *The lls1 phenotype*.
(A) 1.Tomato plant showing *lls1* like symptoms as a result of VIGS 4 weeks after in planta *Agrobacterium tumefaciens* transformation. The stem and fruits remain unaffected. 2.Tomato leaflets at different stages of development of the *lls1* phenotype. The progression of the *lls1* phenotype is given from left to right. The first spots appear at the tips of the leaflets. The tissue between the lesions dries rapidly after the spots spread and finally, the whole leaflet wilts and dies. 3. From left to right tomato leaflets from a control plant, from a plant inoculated with *A. tumefacien*s bearing pGR106 (PVX) and pGRtlls6/7 (pGR106::tomato *Lls1* RT-PCR product).
(B) 1.*N. benthamiana* control plant. 2. *N. benthamiana* inoculated with *A. tumefacien*s bearing: pGR106 (PVX). 3. *Nicotiana benthamiana* inoculated with *Agrobacterium tumefacien*s bearing pGRtlls6/7 (pGR106::tomato *Lls1* RT-PCR product). The pictures of *N. benthamiana* plants are taken 4.5 weeks post *Agrobacterium* transformation.
On the enlarged panels A1 and B3 the typical zoned pattern is visible.
Results presented in (A) and (B) are representative for five independent experiments in tomato (15 plants per experiment) and three independent experiments in *N. benthamiana* (10 plants per experiment).
(C) Light influence on the tomato *lls1* phenotype. The shown tomato leaflets are from plants inoculated with pGRtlls6/7 (pGR106::tomato *Lls1* RT-PCR product). (1) A tomato leaflet with four (see the yellow arrows) *lls1* like lesions before the plant was placed for 48h in dark. (2) The leaflet as in (1), after 48h of darkness. (3) A tomato leaflet with *lls1* like lesions. (4) The leaflet as in (3), after 48h under normal greenhouse light conditions.

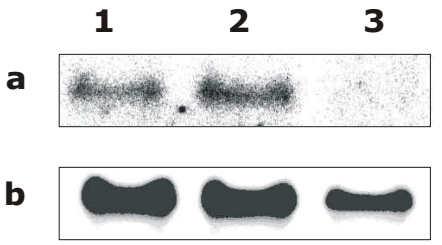


**Figure 4**. *Northern blot analysis of tomato plants with and without the silencing construct.*
Total RNA was extracted from tomato leaflets at the stage shown in Figure 3(A3). (1) Tomato leaflets from unaffected plants, (2) and (3) from the plants inoculated with *Agrobacterium tumefaciens* bearing pGR106 (PVX) and pGRtlls6/7 (pGR106::tomato *LLS1* RT-PCR product), respectively.
Hybridisation was performed with the (a) tomato *Lls1* 5'RACE probe; (b) *Arabidopsis* 18S rRNA probe.

to the other plants from the same inoculation experiment. While kept in dark conditions the tomato leaflets did not form new lesions (Figure 3C1 and 3C2).

## Discussion

In order to obtain a lesion mimic phenotype in tomato, we used the high sequence similarity between the maize and the *Arabidopsis Lls1* homologues (Gray *et al.*, 1997). The tomato *Lls1* homologue we cloned confirmed the expected high sequence identity with *Lls1* from maize and *Arabidopsis*, about 70% at the nucleotide level and about 75-80% at the protein level. The Southern blot (Figure 1) and blast results we obtained are consistent with there being only one copy of the *Arabidopsis Lls1* gene and most likely only one copy in tomato as well. As a result of silencing, we obtained in tomato and in *N. benthamiana* phenotypes (Figure 3) resembling *lls1* in maize (Gray *et al.*, 1997; Ullstrup and Troyer, 1967). The loss of function of *Lls1* homologues resulted in a similar phenotype in monocotyledons (maize) and dicotyledons (tomato, *N. benthamiana*) suggesting they are functionally conserved in these plants species.

The EST data and the results from (Simmons *et al.*, 1998) indicate that the *Lls1* gene is expressed in different plant tissues and cells. However, the silencing of the gene resulted in a phenotype that affected only the leaf tissue in tomato, *N. benthamiana* (Figure 3) and maize (Ullstrup and Troyer, 1967). This indicates that proper functioning of the *Lls1* gene is essential for survival of the cells in the leaves but not in the stems for example.

Southern blot analysis among dicots (Figure 1), EST data and the results of (Gray *et al.*, 1997) showed that homologues of the *Lls1* gene are present in all studied plants. Database searches did not reveal homologous protein sequences from animal species. The most closely related proteins also sharing the same domain structure with the LLS1 proteins (Figure 2a) were from photosynthesising organisms, a putative protein from *Synechocystis sp.* (slr1747) and TIC55 from *Pisum sativum*. The first protein is from a photosynthetic *Synechocystis sp.* and the second is part of the chloroplast inner envelope protein import machinery (Caliebe *et al.*, 1997). Other proteins that show high homology (Figure 2b) are not only the α-subunits of

ARH dioxygenases involved in the degradation of phenolic compounds or demethylases in bacteria, but CBSes and CAOs as well, which are involved in chlorophyll b formation (Tanaka *et al.*, 1998; Tomitani *et al.*, 1999). After light absorption, both chlorophyll a and b and many intermediates of their biosynthetic pathway may interact with oxygen and produce singlet oxygen instead of transferring the excitation energy to their ultimate targets (Reinbothe *et al.*, 1996). Two other lesion mimic phenotypes, *les22* from maize and *lsd1* from *Arabidopsis* are light dependent and elevated levels of ROI are reported in both prior to death of the cells (Dietrich *et al.*, 1997). In our experiments, in the absence of light the *lls1* phenotype developed slowly, therefore light is enhancing the lesion development. Also, we did not observe formation of new lesions when plants were placed in dark for 48h (Figure 3C), indicating that light is important for the initiation of the cell death process in *lls1* mutants. It is possible that the LLS1 gene product is involved in the protection of the photosynthesizing cells from higher levels of ROI, which if not controlled properly triggers the cell death process.

Cloning genes, which when mutated result in a lesion mimic phenotype, provides a clue as to how the process of cell death is regulated in plants. Characterizing homologous genes in different plant species helps to understand their conservation of function in the plant kingdom. In our study, we obtained a tomato lesion mimic phenotype via silencing of the homologous gene causing the lesion mimic phenotype in maize. The engineered tomato phenotype is functionally similar to the maize phenotype. This supports the hypothesis that, at least in some parts, the mechanism of cell death induction in plants is conserved.

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